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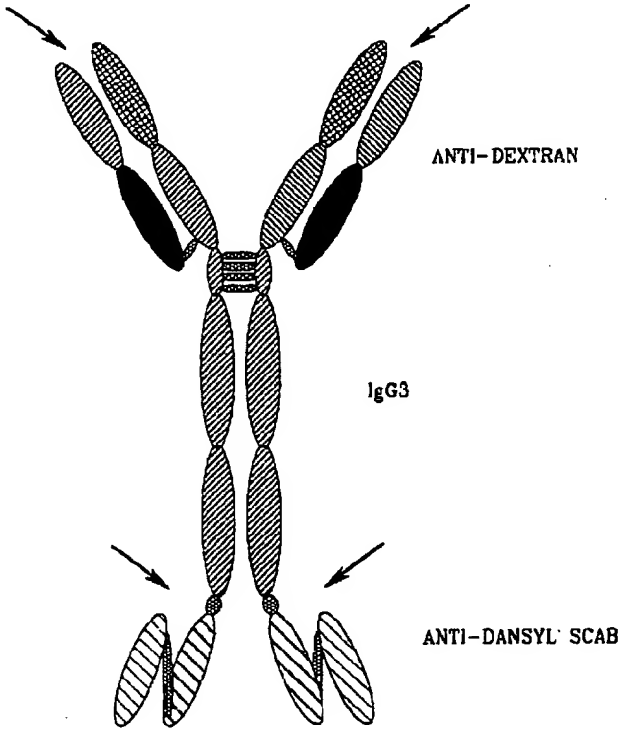
(54) Title: GENETICALLY ENGINEERED BISPECIFIC TETRAVALENT ANTIBODIES

(57) Abstract

The invention relates to a method for the production of recombinant bispecific tetravalent antibodies. These antibodies are useful in targeting toxins and activated T cells to tumor cells as well as in immunodiagnostics. These antibodies are constructed by fusing a DNA segment encoding a single chain antibody with a DNA segment encoding an IgG constant region. This fusion is then ligated to a DNA segment encoding a heavy chain variable region with different specificity. Cells are cotransfected with this construct and a vector encoding a light chain variable region having the same specificity as the heavy chain variable region.



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GENETICALLY ENGINEERED BISPECIFIC TETRAVALENT ANTIBODIES

GOVERNMENT SUPPORT

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FIELD OF THE INVENTION

This invention relates to the production of bispecific, tetravalent antibodies using recombinant DNA methods. The antibody protein is produced at the DNA level and expressed by transfection. The resulting antibody has two different specificities, one at the N-terminus and one at the C-terminus. This invention may have both therapeutic and immunodiagnostic applications.

BACKGROUND OF THE INVENTION

The development of monoclonal antibody technology has resulted in an explosion of research into the diagnosis and treatment of cancer and other disorders of the immune system. However, the use of murine monoclonal antibodies in humans has had limited success, mainly due to development of a human anti-mouse antibody response accompanying the long-term use of these molecules (Schroff et al., (1985) *Cancer Res.*, **45**: 879; Steinitz et al., (1988) *J. Immunol.*, **141**: 3515).

To make murine monoclonal antibodies less antigenic to humans, investigators have engineered antibodies containing human framework sequences in the majority of the molecule but murine sequences in the complementarity determining regions (CDRs) of the heavy and light chain hypervariable regions or antibodies with murine variable regions and human constant regions (Boulianne et al., (1984) *Nature*, **312**: 643-646; Neuberger et al., (1985) *Nature*, **314**: 268-270; Morrison, (1992) Annu. Rev. Biochem., **10**: 239-265). Another approach to producing more effective monoclonal antibodies is the conjugation of chemotherapeutic drugs and other toxins in order to effectively target these molecules to cancer cells (Krolick et al., (1992) *J. Exp. Med.*, **155**: 1797; Vitetta et al., (1983) *Science*, **219**: 644; Nolan et al., (1992) *Intl. J. Clin. Leb. Res.*, **22**: 21).

Another potential means of producing more effective antibodies is the development of bispecific molecules capable of simultaneously binding two different antigens (Wong and Colvin, (1987) *J. Immunol.*, **139**: 1369). These antigens may be, for example, a tumor cell antigen and an effector cell such as an

activated T cell or functional agent such as a cytotoxin (Flavell et al., (1991) *British J. Cancer*, **64**: 274; Runge et al., (1990) *Bioconjugate Chem.*, **1**: 274). Such tumor cell antigens include carcinoembryonic antigen, a widely-expressed tumor cell marker and Le^y antigen expressed on breast, lung and colon carcinomas. This provides an effective means to facilitate targeting of these cells and toxins to transformed cells. This method has been used to increase cytotoxicity of activated T cells toward human glioma cells (Nitta et al., (1990) *J. Neurosurg.*, **72**: 476-481; Nishimura et al., (1992) *J. Immunol.*, **148**: 285-291) and to target the toxins ricin and vindesine to tumor cells (Embleton et al., (1991) *British J. Cancer*, **63**: 670-674; Corvalan and Smith, (1987) *Cancer Immunol. Immunother.*, **24**: 127-132). Finally, catalytic antibodies have also been described which may act as highly selective catalysts in biology, chemistry and medicine (Shokat and Schultz, (1990) *Annu. Rev. Immunol.* **8**: 335-363).

Bispecific antibodies have been produced using a variety of methods. Univalent fragments of pepsin-treated antibodies of different specificities or of complete antibodies have been chemically linked to produce bispecific antibodies (Raso and Griffin, (1981) *Cancer Res.*, **41**: 2073; Glennie et al., (1987) *J. Immunol.*, **139**: 2367; Glennie et al., (1988) *J. Immunol.* **141**: 3662-3670). In addition, fusion of two hybridoma cells yields a hybrid hybridoma called a quadroma which produces some mixed molecules having the specificities of each hybridoma (Reading, (1983) in *Hybridomas and Cellular Immortality*, B. H. Tom and J. P. Allison, eds., p.235, Plenum Press, New York; Milstein and Cuello, (1984) *Immunol. Today*, **5**: 299; Kontsekova et al., (1992) *Hybridoma*, **11**: 461; Lloyd et al., (1991) *J. Nat. Med. Assoc.*, **83**: 901). Genetically engineered single chain antibodies against molecules including bovine growth hormone, digoxin and fluorescein have also been produced (Bird et al., (1988) *Science*, **242**: 423-426; Huston et al., (1988) *Proc. Natl. Acad. Sci. USA*, **85**: 5879-5883; Bedzyk et al., (1990) *J. Biol. Chem.*, **265**, 18615-18620).

Unfortunately, all of the aforementioned approaches have certain disadvantages and limitations. Chemical modification of antibodies is tedious and can lead to side reactions which damage the antigen combining site. Quadroma production has proven inefficient due to the difficulty in separating the bispecific antibodies from the diverse population secreted by the original two hybridomas. Finally, single chain antibodies without constant regions also lack F_c-mediated effector functions such as F_c receptor binding and complement fixation.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the primers and PCR reactions used to generate the anti-dansyl single chain antibody. Primers A and B were used to amplify V_H and to incorporate restriction sites and linkers. Primers C and D were used to amplify V_L. In a second round of PCR the products of the previous reaction were used as templates. Primers A and D were used to amplify and join the V_H and V_L to produce the SCAB. The number of nucleotides in the primer and their origin are indicated below the arrows. X = extra nucleotides.

Figure 2 illustrates the construction of the pAH4633 expression vector expressing the heavy chain of the bispecific antibody. The PCR generated single chain antibody was subcloned into pSK3461, an intermediate vector containing a human IgG3 constant region. The BamH1-Sal1 fragment containing the constant region of the SCAB from pSK4629 replaced the same fragment from pAH3617, an anti-dextran V_H expression vector to generate pAH4633.

Figure 3 is a schematic representation of the expected structure of the bispecific antibody. An anti-dansyl SCAB was fused to the 3' end of an anti-dextran IgG3 chimeric antibody. The arrows indicate the antigen combining sites.

Figure 4 indicates a half-life determination of the bispecific antibody. 1 x 10^6 counts of [^{125}I] labeled antibody were injected intraperitoneally into mice in triplicate and whole body counts were measured. The time, in hours, is indicated on the x-axis and the % of cpm remaining is indicated on the y-axis. TAAM = SP2/0 transfectant; TPO = IgG3 chimeric wild type antibody. The half-life of each antibody, in days, is indicated.

Figure 5 illustrates a high affinity F_c receptor binding assay. ^{125}I labeled antibodies were incubated in the presence of varying amounts of excess cold ligand with 2 x 10^6 interferon γ-treated U937 cells for 3 hours at 14°C. A 500 fold excess of Miles gamma globulin was used to determine nonspecific binding. Specific binding is plotted as a percentage of the total number of counts. The fold excess of cold ligand is indicated on the x-axis and the % of specific binding is indicated on the y-axis.

Figure 6 is a bar graph showing the binding of C1q to antigen-antibody coated plates. C1q binding was detected by a sandwich ELISA with goat anti-human C1q antibody and swine anti-goat alkaline phosphatase-conjugated secondary antibody. The absorbance at 410 nm is shown on the y-axis.

SUMMARY OF THE INVENTION

One embodiment of the present invention is a method for the production of a bispecific, tetravalent antibody by the steps of:

producing a DNA segment encoding a single chain antibody (SCAB) consisting of fused DNA segments encoding variable heavy and light (V_H and V_L) immunoglobulin chains;

ligating the SCAB DNA segment to a DNA segment encoding an immunoglobulin G (IgG) constant region;

constructing an expression vector containing the SCAB-constant region ligation fused to a DNA segment encoding a V_H region having a different specificity; and

cotransfecting and expressing the vector and a vector containing a DNA segment encoding the V_L region of the same specificity as the second V_H region into cells.

Preferably, in this method, the SCAB has the anti-dansyl V_H and V_L regions, is produced by PCR overlap extension and the V_H-V_L fusion is through a plurality of gly gly gly gly ser linkers. Advantageously, the IgG constant region is human and the expression vector is eukaryotic. Most preferably, the eukaryotic expression vector is pAH4633. In addition, the V_H and V_L specificities are preferably anti-dextran and the cells are mammalian. Most preferably, these mammalian cells are murine SP2/0 cells.

Still another embodiment of the present invention consists of an isolated recombinant, bispecific antibody.

A further embodiment is an expression vector encoding the bispecific antibody consisting of:

DNA segment I encoding the V_H and V_L regions joined by a flexible linker;

DNA segment II encoding a human IgG3 constant region fused to DNA segment I; and

DNA segment III encoding a V_H region with a different specificity from the V_H region of DNA segment I.

The present invention also embodies a transformed cell line continuously expressing the bispecific antibody.

Yet another embodiment of the present invention is a method for targeting immune cells to tumor cells by the steps of:

contacting the immune cells with a recombinant tetravalent antibody

having affinity for an antigen expressed on the immune cell surface on a first end of the antibody and affinity for a tumor cell surface antigen on a second end of the antibody; and

administering the immune cell-antibody complex to tumor cells.

Another aspect of the present invention includes activating the immune cells with a cytokine prior to the formation of an immune cell-antibody complex and injection into a mammal.

A further aspect of the present invention includes directly injecting the aforementioned recombinant tetravalent antibody *in vivo* wherein the antibody is not complexed to an immune cell prior to injection.

Preferably, the immune cells are either cytotoxic T-cells or lymphokine activated killer cells administered *in vitro* and the cytokine is either IL-1, IL-2, IL-6, interferon α or interferon γ. Most preferably, the cells are administered *in vivo*.

An even further embodiment of the present invention is a method for targeting toxins or chemotherapeutic agents to tumor cells by the steps of:

contacting the agent with a recombinant tetravalent antibody having anti-toxin specificity at a first end of the antibody and affinity for a tumor cell antigen at a second end of the antibody; and

administering the toxin-antibody complex to tumor cells.

Preferably, the toxin is either ricin, adriamycin, daunomycin, vindesine, doxorubicin or taxol and the tumor cells are *in vitro*. Most preferably, the tumor cells are *in vivo*.

Finally, another embodiment of the present invention is a method of identifying antigens present in a biological sample or expressed on the cell surface by the steps of: isolating a biological sample of interest;

contacting the biological sample with an isolated bispecific antibody having affinity for an antigen of interest at one of its ends and affinity for a adiolabeled compound, dye or enzyme able to catalyze a colorimetric reaction at its other end; and

detecting the binding of the antibody to the antigen of interest.

DETAILED DESCRIPTION OF THE INVENTION

We describe a new method of producing bispecific, tetravalent antibodies using recombinant DNA technology. Our approach is the production of a single chain antibody (SCAB; Chaudary et al., (1990) *Proc. Natl. Acad. Sci. USA*, **87**: 1066; Ayala et al., (1992) *BioTech*, **13**: 790) and its fusion to a complete antibody

having a different specificity. This gene fusion is expressed by transfection resulting in a tetravalent antibody having dual specificity. The advantages of this approach over the prior art include production of a homogeneous antibody population, all molecules having two sites which bind one antigen and two sites which bind a different antigen. Additionally, since the constant region of the antibody is preserved, the molecule can bind to F_c receptors on monocytic cells. Finally, the antibody may be more effective at inducing target cell lysis due to the presence of twice as many binding sites for each antigen, thus increasing its avidity for both target and effector cells.

Bispecific, tetravalent antibodies have the ability to bind a total of four antigens of two different types (two at each end). The antibodies are produced by combining a DNA sequence encoding a SCAB with a DNA sequence encoding the heavy chain region of a different antibody. Normally these sequences are combined such that the constant region of the heavy chain antibody is linked to the variable region of the SCAB through a short flexible linker region. The nucleotide sequences encoding the heavy chain and the SCAB are advantageously inserted into an expression vector and transfected into a cell line capable of expressing the protein product. Cells transfected with the aforementioned construct are cotransfected with a separate vector encoding the light chain corresponding to the aforementioned heavy chain. In this manner, the transfected cells produce a single protein species comprising the heavy chain linked to the SCAB and a second protein comprising the light chain. These proteins assemble to produce functional antibodies and are secreted.

This method imparts predictability to the process of producing bispecific antibodies. A SCAB with a known specificity and a heavy chain from a second antibody, also having a known specificity, can be combined to predictably produce a tetravalent, bispecific antibody. This predictability is very advantageous in view of the prior art methods of producing bispecific antibodies.

One of ordinary skill will also appreciate the possibility of engineering, using similar methods, a tetravalent, tetraspecific antibody able to bind four antigens, each having a different specificity, a tetravalent, trispecific antibody able to bind one antigen at one of its ends and two antigens at its other end and a tetravalent monospecific antibody able to bind four antigens of a particular type. A tetravalent, monospecific antibody will advantageously increase the avidity of antigen binding and will be especially useful for immunodiagnostics. In addition,

catalytic antibodies can be generated by the method of the present invention and targeted to an antigen such as a tumor cell marker. The other end of the molecule comprises an antibody having catalytic activity. For example, the catalytic antibody may act as a protease which, once inside the target cell, will hydrolyze essential proteins resulting in cell death.

Although the two specificities of the antibody described in the present invention are directed towards dansyl and dextran, these specificities are only illustrative. Using this technology, one of ordinary skill in the art will be able to engineer antibodies having a wide variety of specificities. The recombinantly-expressed, isolated antibodies will be particularly useful for targeting activated immune cells to tumor cells by simultaneously binding antigens on the tumor cells and effector cells, thus bringing the cells in close proximity and resulting in tumor cell lysis. In a similar fashion, the antibody can also be engineered to bind a toxin or chemotherapeutic agent at one of its ends and a tumor cell antigen at its other end, also resulting in tumor cell lysis by specifically delivering the toxin to the tumor cell. Due to the immunological specificity of the delivery mechanism, normal cells will not be appreciably affected.

The antibodies of the present invention will also be useful as immunodiagnostic agents in the detection of a number of pathological conditions including cancer, AIDS and bacterial infections. The antibody molecules can be engineered to have an affinity for tumor cell markers, HIV envelope glycoproteins such as gp120, bacterial membrane proteins or viral surface proteins such as influenza hemagglutinin at one end and an affinity for an enzyme able to catalyze a colorimetric reaction at the other end.

Although one means of expressing the recombinant antibody is by insertion into the eukaryotic expression vector pAH4633 and expression in murine cells, other eukaryotic expression vectors and eukaryotic cell types known by those of ordinary skill in the art are also within the scope of the present invention. It is also envisioned that one of ordinary skill will be able to activate immune cells with other molecules in addition to IL-1, IL-2, IL-6 or the interferons. These other activating molecules, as well as other types of activated immune cells, are also within the scope of the present invention as a means of targeting activated immune cells to tumor cells. In addition, it will be appreciated that toxins and chemotherapeutic agents other than those specifically mentioned can also be bound by the bispecific antibodies of the present invention.

It is also envisioned that in the use of the antibody of the present invention as an immunodiagnostic agent, a number of detection systems could be used including, but not limited to, colorimetric labels, radiolabels and dyes.

As a first step in engineering a recombinant bispecific tetravalent antibody, an anti-dansyl single chain antibody was constructed as described in the following example.

Example 1

Construction of an anti-dansyl single chain antibody

An anti-dansyl single chain antibody (SCAB) was produced using PCR to join two variable regions by overlap extension (Ho et al., (1989) *Gene*, 77: 51). A flexible linker region consisting of three gly gly gly gly ser repeats was used to connect the C-terminus of the heavy chain variable region (V_H) and the N-terminus of the light chain variable region (V_L) in order to maximize flexibility while minimizing steric hindrance during protein folding (Huston et al., (1988) *Proc. Natl. Acad. Sci. USA*, 85: 5879-5883).

For the PCR reaction, the 3' primer for V_H (B) (SEQ ID NO: 1) and the 5' primer for V_L (C) (SEQ ID NO: 2) were designed to have a complementary region in the linker portion. The V_H and V_L regions were independently amplified, followed by a second PCR reaction in which these products were used as templates. The complementary sequences in the linker hybridized, joining the V regions, and the primers A (SEQ ID NO: 3) and D (SEQ ID NO: 4) were then used to amplify the SCAB (Figure 1). The 5' primer used to amplify V_H was designed to eliminate a BamHI site present in amino acid 16 of framework 1 (FR1). With this approach, amplification and mutagenesis were reduced to a single step.

Briefly, 50 ng of plasmid containing a cloned anti-dansyl V_H or V_L was used as a template for PCR. The primers used are shown in Table 1. PCR reactions were performed in a final volume of 100 μl in 10 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl, 100 μg bovine serum albumin and 2.5 units *Taq* polymerase for 25 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The size of the PCR products was verified by agarose gel electrophoresis and staining with ethidium bromide.

In the second PCR reaction, 5 μl of each first reaction was used as a template. The primers used were 5'A (SEQ ID NO: 3) and 3'D (SEQ ID NO: 4). The buffer and primer concentrations were the same as above. The PCR parameters were 1 min at 94°C, 1.5 min at 60°C, 72°C for 2 min for 25 cycles and

a 10 min extension at 72°C. Both the variable region fragments and the SCAB were cloned into plasmid Bluescript T-A (blunt ended by digestion with Eco RV and tailed with deoxythymidine triphosphate using terminal transferase (Holton and Graham, (1990) *Nucl. Acids Res.*, **19**: 1156)). The plasmid was used to transform competent *E. coli* XL1-blue cells (Stratagene). White colonies containing inserts were picked, miniprep DNA was analyzed, and inserts were sequenced using Sequenase (United States Biochemical) according to the manufacturer's instructions. Several independent clones from different PCR reactions were sequenced in both directions (Figure 2; SEQ ID NO: 5). The sequences were analyzed for PCR-induced mutations, verification of correct joining of the variable regions and for the presence of restriction sites. Large preparations of the correct clones were prepared using the maxi DNA prep kit (QIAGEN). Although the SCAB was constructed in the order V_H-linker-V_L, the reverse construct V_L-linker-V_H is also within the scope of the present invention.

The SCAB was then fused to a human IgG3 constant region to produce a recombinant bispecific antibody as described in the following example.

Example 2

Construction of the bispecific antibody expression vector

The anti-dansyl SCAB contained a PvuII site at its 5' end and an EcoRI site at its 3' end. There were 14 additional base pairs upstream of the first codon of FR1 which provided a short flexible linker and allowed the cloning of the SCAB in frame with a human IgG3 constant region (in plasmid pSK3461) that had been mutagenized at its 3' end to introduce a SspI site by adding TATT downstream of the last amino acid of C_H3. The resulting AATATT site, when cut, provided two extra bases (GT) that maintained the reading frame in the linker region. pSK3461 also contained a linker region 3' of the SspI site with an EcoRI site. A polyadenylation site was 3' of the EcoRI site. Cloning into the SspI and EcoRI sites thus provided a constant region fusion containing a polyadenylation signal (Figure 2).

To clone the SCAB into the above vector, a complete EcoRI digestion and a partial SspI digestion were performed. The 6.6 kb vector was ligated to the 770 base pair SCAB and the plasmid was used to transform competent *E. coli* HB101 cells. Positive clones were screened by restriction analysis of miniprep DNA. The final 7.4 kb construct was called pBSK4629 (Figure 2).

pAH3617 is a 14 kb eukaryotic expression vector which contains the anti-

dextran V_H chain, human IgG3 constant region, and SV40 promoter and enhancer 5' of the V_H chain. To replace the constant region of this vector with the constant region containing the downstream anti-dansyl SCAB, both plasmids pSK4629 and pAH3617 were cut with BamHI and SalI. The 4.4 kb SalI-BamHI piece from pBSK4632, including the human IgG3 constant region, and the anti-dansyl SCAB containing a stop codon and a polyadenylation signal, were ligated to the expression vector thus replacing the original constant region (Figure 2). The final expression vector, pAH4633, contained an SV40 promoter and enhancer, the anti-dextran V_H region, the human IgG3 constant region joined to the anti-dansyl SCAB, a histidinol eukaryotic selectable marker and an ampicillin prokaryotic selectable marker.

Although the SCAB was joined to the CH_3 region of the heavy chain antibody, one of ordinary skill in the art will appreciate that the SCAB can also be joined to the heavy chain antibody at other positions including after CH_1, after the hinge region and after CH_2. Vectors are available which will facilitate these combinations and the resulting recombinant antibodies will also be bispecific.

To produce a complete bispecific tetravalent antibody (Figure 3), it was necessary to cotransfect both the heavy and light chain Ig vectors as described in the following example:

Example 3

Transfection and Selection

The light chain expression vector pAH3634 contained the anti-dextran V_L region, the human kappa constant region and an SV40 promoter and enhancer.

Prior to transfection, 10 μg of pAH4633 and pAH3634 DNA were linearized using BspCI (blunt cutter) which cuts within the ampicillin resistance gene. The nonproducing murine myeloma cell line SP2/0 was used as a recipient in independent transfections.

For electroporation, 2×10^6 cells in log phase were washed twice with cold phosphate buffered saline (PBS) and resuspended in 0.9 ml of the same buffer containing the linearized vector. The cells were incubated in a 0.4 cm gap electroporation cuvette (Bio-Rad Laboratories) for 10 min on ice. Using a Gene Pulser (Bio-Rad), an electrical pulse of 200 volts and 900 microfarads was delivered and the cuvette was incubated as above. The cells were washed in cold Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS) and resuspended at 10^5 cells/ml in IMDM containing 10%

FCS, 100 units/ml nystatin and 100 μg/ml gentamycin. The cells were plated in 96 well microtiter plates at 100 μl/well. After two days 100 μl of selection medium containing 5 mM histidinol was added to each well.

Positive clones were detected by enzyme-linked immunosorbent assay (ELISA) of the cell culture supernatants using 96 well dansyl-BSA-coated microtiter plates. The highest producing clones were expanded to 5 and 25 ml petri dishes. The transfectant in SP2/0 was named TAAM and exhibited dansyl binding.

To determine whether the recombinant antibodies were correctly assembled and of the expected size, secreted antibodies were analyzed as described in the following example.

Example 4

Analysis of antibodies in cell culture supernatants

Transfectants were metabolically labeled with ^{35}S methionine overnight, centrifuged and the supernatant analyzed for the presence of secreted Ig. To analyze intracellular Ig, cytoplasmic extracts were prepared by lysing the cells with 0.5% NP-40 in isotonic buffer. Cytoplasmic and secreted antibodies were immunoprecipitated with rabbit anti-human constant region antiserum followed by addition of *Staphylococcus aureus* protein A for 15 min at 4°C. The samples were layered over a 30% sucrose pad containing NDET (10 mM Tris-HCl, pH 7.4, 1% NP-40, 0.4% sodium deoxycholate, 66 mM EDTA), centrifuged for 5 min, and the supernatant discarded. The pellet was washed once with water, resuspended in sample buffer (25 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.008% bromphenol blue) and boiled for 2 min. To analyze the size of the protein synthesized by the transfectants, immunoprecipitates were analyzed on 12% Tris-glycine SDS gels after reduction with 2-mercaptoethanol. The assembly patterns and polymerization state of the Igs were determined by fractionating the immunoprecipitates on 5% phosphate gels under nonreducing conditions. The transfectants produced a secreted Ig of 226 kDa, the size expected for the construct.

To determine whether the secreted bispecific antibodies were N-linked glycosylated, transfectants were metabolically labelled as described above but in the presence of tunicamycin, an inhibitor of N-linked glycosylation. The labeled antibody was immunoprecipitated and analyzed by SDS polyacrylamide gel electrophoresis. The results indicated that the antibodies were N-linked glycosylated since when grown in the presence of 8 mg/ml tunicamycin the

transfectants secreted heavy chains with a decreased apparent molecular weight.

To obtain larger amounts of antibody, a large scale protein purification was performed as described in the following example.

Example 5

Protein purification

After verifying the correct nature of the secreted antibodies, cells were expanded in tissue culture roller bottles containing 1% alpha calf serum in IMDM. Four liters of cell culture supernatant from each clone were recovered and antibodies were purified on an affinity column containing a dansyl isomer coupled to Sepharose. The antibodies were eluted with a second dansyl isomer, which binds to the antibodies, thus competitively displacing them from the immobilized dansyl isomer, dialyzed against tris-saline and concentrated by vacuum. Purified antibodies were analyzed on 12% tris-glycine SDS gels for reduced samples or on 5% phosphate gels for unreduced samples as described above. The purified protein was identical in molecular weight to the biosynthetically labeled protein described in Example 4.

Both the screening assay and the affinity purification demonstrated the affinity of the antibody for the dansyl epitope. To determine the antibody exhibited dextran specificity, a competition ELISA was performed by well known methods. The results indicated an apparent association constant of 5×10^6, slightly less that the affinity seen for a wild type anti-dextran IgG3 with the same V_H region. Now that the bispecific nature of the antibody had been demonstrated, we examined its half-life *in vivo* as described in the following example.

Example 6

Half-life studies

BALB/c mice (4-6 weeks old) were administered 10 mg/ml potassium iodide in water for 6 days prior to the experiment in order to saturate the thyroid with cold iodine to reduce any background counts. [^{125}I] labeled protein (1×10^6 cpm of TAAM, TPO, and BSA) in 250 μl sterile PBS was injected intraperitoneally into mice in triplicate. Whole body counts were taken at various time points for a total of 400 hours to determine the alpha and beta phase of the count decay. The half-life of the protein was calculated from the slope of the beta curve. The TAAM antibody was found to have a 1.6 fold longer half life than wild type IgG3 (Figure 4).

To determine whether the constant region of the TAAM recombinant

antibody was able to bind to F_c receptors, a cell binding assay was performed as follows.

Example 7

F_c receptor binding assay

Human U937 monocytic cells expressing the high affinity $F_c\gamma RI$ receptor were grown in RPMI containing 10% FCS and stimulated with 100 units/ml human interferon γ for 2-3 days prior to the assay. Cells were harvested by centrifugation at 1,000 rpm for 5 min, washed in cold PBS and counted using a hemocytometer. The cells were resuspended at 1 x 10^7 cells/ml in serum-free DMEM and incubated for 2 hours at 37°C. Eppendorf tubes were prepared which contained a constant amount of [^{125}I] labeled recombinant antibody (approximately 20 ng; 1 x 10^5 cpm) in 50 ml binding buffer (0.1 M HEPES, pH 7.4-7.7, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 15 mM acetic acid, 10 mM glucose, 1% BSA).

Fifty μl of the above solution containing varying concentrations of the same unlabeled protein as competitor were added to duplicate tubes. Cells were resuspended in binding buffer at 4-6 x 10^6/ml and 400 μl total volume was added to each tube. Samples were incubated for 3 hours at 14°C with constant rotation, pelleted, the pellets carefully washed with cold PBS and counted for 2 min in a gamma counter. The results indicated that the antibody had an affinity for the F_c receptors on U937 cells (Figure 5) with a binding dissociation constant of around 5 x 10^{-7} M.

To evaluate another function associated with the constant region of antibodies, C1q binding, the first step in the activation of the complement cascade, was evaluated as described below:

Example 8

C1q binding assay

Immulon-2 microtiter plates (American Scientific) were coated with 100 μl dansyl-coated BSA (substitution ration 40:1; 10 mg/ml in HEPES-buffered saline (HBS)) per well, incubated at 4°C overnight and washed 6 times with HBS containing 0.02% sodium azide. Plates were blocked with 200 μl 3% BSA in HBS. After blocking, 20 μg of either bispecific or control antibody in HBS (containing 1% BSA) was added (100 μl/well) in quadruplicate and incubated for 2 hours at room temperature. Wells were washed 6 times with HBS and 100 μl 0.25% normal human serum was added for 2 hours at 37°C. Wells were washed and incubated with 100 μl goat anti-human C1q (1:10,000; Atlantic Antibodies) for 1 hour at

37°C. Wells were washed to remove unbound antibodies followed by incubation with 100 μl/well alkaline phosphatase-conjugated swine anti-goat antibody (1:20,000) for 2 hours at 37°C. Wells were washed to remove unbound antibody and 100 μl p-nitrophenyl phosphate (0.5 mg/ml; Sigma) was added and the absorbance read at 410 nm.

The results indicated that the TAAM antibody bound C1q, although with a decreased efficiency compared to TPO wild type antibody (Figure 6). Since it has been demonstrated that tetravalent, bispecific antibodies can bind F_c receptors and bind C1q, the first step in complement activation, we anticipate that these antibodies will be useful as therapeutics as described below.

Example 9

Use of tetravalent antibody conjugates to lyse tumor cells

Recombinant bispecific monoclonal antibodies having both anti-CD3 or anti-IL-2 receptor (found on activated T-cells) and anti-tumor cell antigen affinities are produced using the method described in Examples 1-3. Other T cell surface antigens are also within the scope of the present invention. A variety of tumor cell antigens can be targeted including, but not limited to, the 145 kDa cell surface glycoprotein expressed on human gliomas and small lung carcinomas, the carcinoembryonic antigen expressed on a wide range of tumor cells and the Le^y antigen expressed on breast, lung and colon carcinoma.

Peripheral blood mononuclear cells (PBMC) are isolated from the blood of healthy donors or tumor patients by Ficoll-Hypaque gradient centrifugation. The cells are then incubated in culture medium in the presence or absence of 100 units/ml recombinant human IL-2 for 3 days at 37°C. Control cells receive no IL-2. Target tumor cells are labeled with ^{51}Cr and plated in 96 well microtiter plates and about 0.1-about 100 μg recombinant antibody is added to each well for about 30-about 60 min. Activated T-cells are then added for about 4 hours. Cytolysis is assessed by gamma counting of the cell supernatant after centrifugation. The use of other activating cytokines, including but not limited to, IL-1, IL-6, and the interferons is also contemplated.

The use of this procedure *in vivo* is also envisioned. Tetravalent, bispecific antibodies are complexed with T cells. Advantageously, these immune cells may be activated *in vitro* prior to complex formation. The immune cell-antibody complex is then injected into a mammal, preferably a human, in an amount sufficient to promote tumor cell lysis and tumor regression. Most preferably, the tetravalent

antibody is injected by itself whereupon it promotes the union of immune cells and tumor cells *in vivo* without the need for prior immune cell-antibody complex formation. The amount of immune cell-antibody complex or of antibody alone administered will depend on the type and size of the carcinoma, the extent of metastasis, the activation state of the immune cell and the affinity of the antibody for the tumor cell antigen. If metastasis has occurred, the administration of more than one tetravalent, bispecific antibody type is also contemplated. The administration of antibodies, either alone or in complex with immune cells, in conjunction with conventional chemotherapy, is also envisioned. Other immune cell types, including, but not limited to, natural killer (NK) and lymphokine-activated killer (LAK) cells are also within the scope of the present invention.

A similar experiment can be performed using an antibody specific for the same aforementioned anti-T cell antigens and for a toxin or chemotherapeutic agent such as ricin or vindesine, respectively. In this embodiment, the bispecific, tetravalent antibody is necessarily complexed to the toxin or chemotherapeutic agent *in vitro* prior to injection *in vivo*. The bispecific antibody can then deliver such an agent to tumor cells resulting in tumor cell lysis and tumor regression.

The tetravalency of the recombinant antibody provides a greater binding capacity than bivalent recombinant antibodies, since each end of the molecule can bind two antigens thus delivering twice as much chemotherapeutic agent or toxin to the target cell as would a similar bivalent antibody. Thus, the amount of antibody to be administered could be reduced without reducing its efficacy. In addition, since the antigen combining sites are located on opposite ends of the molecule, this provides greater binding flexibility thus allowing the antibody to bridge greater distances between targets.

The antibody of the present invention can also be used in immunodiagnostics as described below.

Example 10

Use of the tetravalent antibody in immunodiagnostics

A bispecific tetravalent antibody is produced by the method described in the examples above having a specificity against both a tumor cell antigen of interest and either a radiolabeled compound, a dye or a protein capable of catalyzing a colorimetric reaction. The labeled antibody can be used to stain cell or tissue sections to determine the presence of a tumor cell antigen. In addition, since certain proteins are released from tumors into the blood, such as prostatic acid

phosphatase from prostate cancer cells, this antibody will be useful in detecting these antigens by enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay, a technique well known in the art. The antibody can also be used to detect viruses and bacteria in body fluids.

While particular embodiments of the invention have been described in detail, it will be apparent to those skilled in the art that these embodiments are exemplary, rather than limiting, and the true scope of the invention is that defined in the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Regents of the University of California

(ii) TITLE OF INVENTION: GENETICALLY ENGINEERED BISPECIFIC TETRAVALENT ANTIBODIES

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Knobbe, Martens, Olson & Bear
(B) STREET: 620 Newport Center Drive, Sixteenth Floor
(C) CITY: Newport Beach
(D) STATE: CA
(E) COUNTRY: U.S.A.
(F) ZIP: 92660

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Altman, Daniel E.
(B) REGISTRATION NUMBER: 34,114
(C) REFERENCE/DOCKET NUMBER: UCLA007.007VPC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (714) 760-0404
(B) TELEFAX: (714) 760-9502

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Primer B

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Primer C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Primer A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AACCTGGAGG TTCCATGAAA 80

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Primer D

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 782 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: anti-dansyl SCAB

(ix) FEATURE:
(A) NAME/KEY: prim_transcript
(B) LOCATION: 12..773

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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GGATGGACTG GGTCCGCCAG TCTCCAGAGA AGGGGCTTGA GTGGGTTGCT GAAATTAGAA 180

ACAAAGCTAA TAATCATGCA ACATACTATG CTGAGTCTGT GAAAGGGAGG TTCACCATCT 240

CAAGAGATGA TTCCAAAAGG AGAGTGTACC TGCAAATGAA CACCTTAAGA GCTGAAGACA 300

CTGGCATTTA TTACTGTACC GGGATCTACT ATCATTACCC CTGGTTTGCT TACTGGGGCC 360

AAGGGACTCT GGTCACTGTC TCTGCAGGTG GCGGTGGCTC GGGCGGAGGT GGGTCGGGTG 420

GCGGCGGATC TGATGTTGTG ATGACCCAAA CTCCACTCTC CCTGCCTGTC AGTCTTGGAA 480

ATCAAGCCTC CATCTCTTGC AGATCTAGTC AGAGCCTTGT ACACAGTAAT GGAAACACCT 540

ATTTACATTG GTACCTGCAG AAGCCAGGCC AGTCTCCAAA GCTCCTGATC TACAAAGTTT 600

CCAACCGATT TTCTGGGGTC CCAGACAGGT TCAGTGGCAG TGGATCAGGG ACAGATTTCA 660

CACTCAAGAT CAGCAGAGTG GAGGCTGAGG ATCTGGGAGT TTATTTCTGC TCTCAAAGTA 720

CACATGTTCC ATTCACGTTC GGCTCGGGGA CAAAGTTGGA AATAAAACGT TAAGAATTCG 780

GG 782

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 253 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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1 5 10 15

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Phe Ser Asp Ala Trp Met Asp Trp Val Arg Gln Ser Pro Glu Lys Gly
            35                  40                  45

Leu Glu Trp Val Ala Glu Ile Arg Asn Lys Ala Asn Asn His Ala Thr
        50                  55                  60

Tyr Tyr Ala Glu Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp
65                  70                  75                  80

Ser Lys Arg Arg Val Tyr Leu Gln Met Asn Thr Leu Arg Ala Glu Asp
                85                  90                  95

Thr Gly Ile Tyr Tyr Cys Thr Gly Ile Tyr Tyr His Tyr Pro Trp Phe
            100                 105                 110

Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala Gly Gly Gly
        115                 120                 125

Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Val Val Met
    130                 135                 140

Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly Asn Gln Ala Ser
145                 150                 155                 160

Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Asn Thr
                165                 170                 175

Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu
            180                 185                 190

Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro Asp Arg Phe Ser
        195                 200                 205

Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser Arg Val Glu
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Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro
225                 230                 235                 240

Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys Arg
                245                 250
```

WHAT IS CLAIMED IS:

1. A method for the production of a bispecific, tetravalent antibody comprising the steps of:

(a) producing a DNA segment encoding a single chain antibody (SCAB) comprising fused DNA segments encoding variable heavy and light (V_H and V_L) immunoglobulin chains;

(b) ligating said SCAB DNA segment to a DNA segment encoding an immunoglobulin G (IgG) constant region;

(c) constructing an expression vector containing said SCAB-constant region ligation fused to a DNA segment encoding a V_H region having a different specificity; and

(d) cotransfecting and expressing said vector and a vector containing a DNA segment encoding the V_L region of the same specificity as said V_H.

2. The method of Claim 1, wherein the DNA encoding said SCAB comprises the anti-dansyl V_H and V_L regions.

3. The method of Claim 1, wherein the DNA encoding said SCAB is produced by PCR overlap extension.

4. The method of Claim 1, wherein said V_H-V_L fusion is through a plurality of gly gly gly gly ser linkers.

5. The method of Claim 1, wherein the DNA sequence encoding said IgG constant region is a human IgG constant region.

6. The method of Claim 1, wherein said expression vector is eukaryotic.

7. The method of Claim 6, wherein said eukaryotic expression vector is pAH4633.

8. The method of Claim 1, wherein said V_H and V_L specificities are anti-dextran.

9. The method of Claim 1, wherein said cells are mammalian.

10. The method of Claim 9, wherein said mammalian cells are SP2/0.

11. An isolated tetravalent, bispecific antibody produced by the method of Claim 1.

12. The isolated tetravalent, bispecific antibody of Claim 11, further comprising a catalytic antibody sequence at one end of said antibody.

13. An expression vector encoding the bispecific antibody of Claim 11, comprising:

(a) DNA segment I encoding the V_H and V_L regions joined by a flexible linker;

(b) DNA segment II encoding a human IgG constant region fused to said DNA segment I; and

(c) DNA segment III encoding a V_H region with a different specificity from the V_H region of said DNA segment I.

14. A transformed cell line continuously expressing the bispecific antibody of Claim 11.

15. A method for identifying antigens present in a biological sample or expressed on the cell surface comprising:

isolating a biological sample of interest;

contacting said biological sample with an isolated antibody according to Claim 11 having affinity for an antigen of interest at first end of said antibody and affinity for either a radiolabeled compound, a dye or an enzyme able to catalyze a colorimetric reaction at second end of said antibody; and

detecting the binding of said antibody to said antigen of interest.

16. The recombinant tetravalent antibody of Claim 11 for use in targeting immune cells to tumor cells *in vivo*.

17. The recombinant tetravalent antibody of Claim 16 in a form suitable for direct injection *in vivo*.

18. The recombinant tetravalent antibody of Claim 11, having anti-toxin specificity at a first end of said antibody and affinity for a tumor cell antigen at a second end of said antibody.

19. The recombinant tetravalent antibody of Claim 11, having affinity for an antigen expressed on the immune cell surface at a first end of said antibody and affinity for a tumor cell surface antigen on a second end of said antibody.

20. Use of a recombinant tetravelent antibody according to Claim 11 in the preparation of a medicament for treatment of tumor cells.

21. A method of targeting immune cells to tumor cells comprising the steps of:

contacting said immune cells with a recombinant tetravalent antibody according to Claim 11 having affinity for an antigen expressed on

the immune cell surface on a first end of said antibody and affinity for a tumor cell surface antigen on a second end of said antibody; and

administering said immune cell-antibody complex to tumor cells.

22. The method of Claim 21, wherein said immune cells are activated with a cytokine.

23. The method of Claim 21, wherein said immune cells are either cytotoxic T-cells or lymphokine activated killer cells.

24. The method of Claim 21, wherein said cytokine is selected from the group consisting of: IL-1, IL-2, IL-6, interferon α and interferon γ.

25. The method of Claim 21, wherein said cells are *in vitro*.

26. The method of Claim 21, wherein said cells are *in vivo*.

27. A method of targeting activated immune cells to tumor cells comprising directly injecting the isolated tetravalent, bispecific antibody of Claim 11 *in vivo*, wherein said antibody is not a part of an immune cell-antibody complex.

28. A method for targeting toxins or chemotherapeutic agents to tumor cells comprising the steps of:

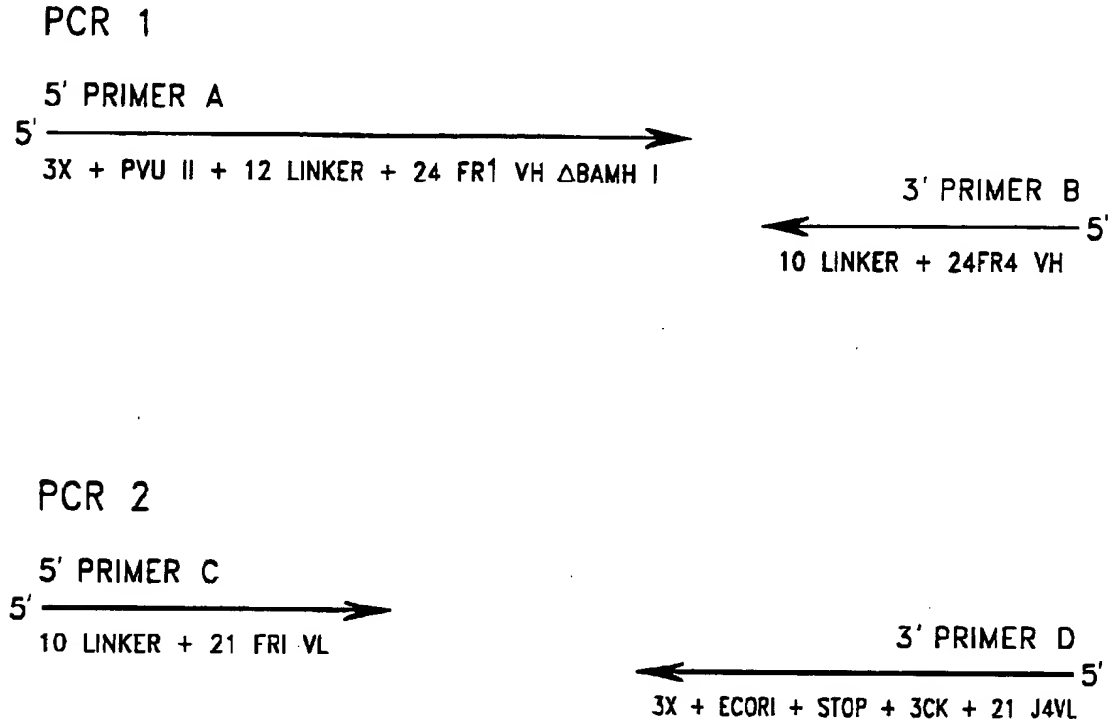
contacting said agent with a recombinant tetravalent antibody according to Claim 11 having anti-toxin specificity at a first end of said antibody and affinity for a tumor cell antigen at a second end of said antibody; and

administering said toxin-antibody complex to tumor cells.

29. The method of Claim 28, wherein said toxin is selected from the group consisting of: ricin, adriamycin, daunomycin, vindesine, doxorubicin and taxol.

30. The method of Claim 28, wherein said tumor cells are *in vitro*.

31. The method of Claim 28, wherein said tumor cells are *in vivo*.


PCR 1
5' PRIMER A
5'
3X + PVU II + 12 LINKER + 24 FR1 VH ΔBAMH I
3' PRIMER B
5'
10 LINKER + 24FR4 VH
PCR 2
5' PRIMER C
5'
10 LINKER + 21 FRI VL
3' PRIMER D
5'
3X + ECORI + STOP + 3CK + 21 J4VL
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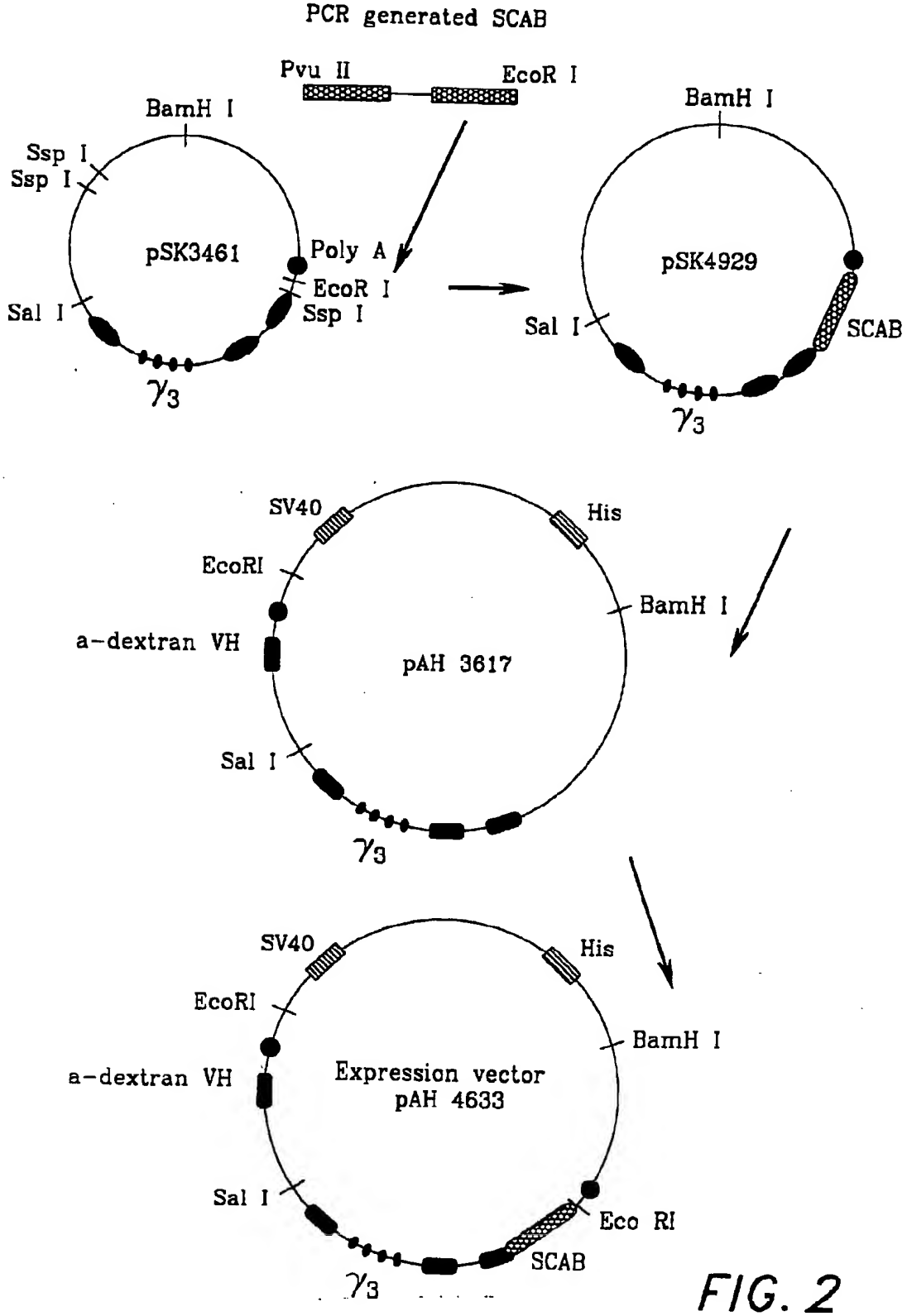
VL
Pvu II
EcoR I

FIG. 1


PCR generated SCAB
Pvu II
EcoR I
BamH I
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Ssp I
pSK3461
Poly A
EcoR I
Ssp I
Sal I
γ3
BamH I
pSK4929
Sal I
SCAB
γ3
SV40
His
EcoRI
BamH I
a-dextran VH
pAH 3617
Sal I
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SCAB
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FIG. 2

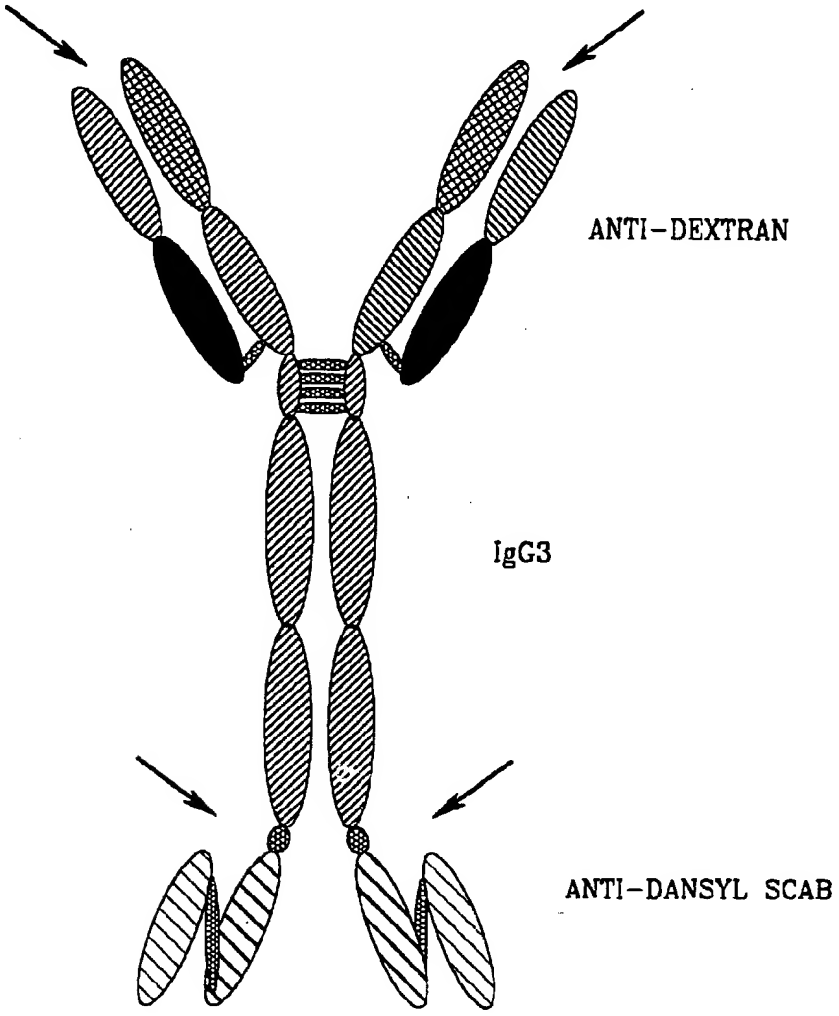

FIG. 3

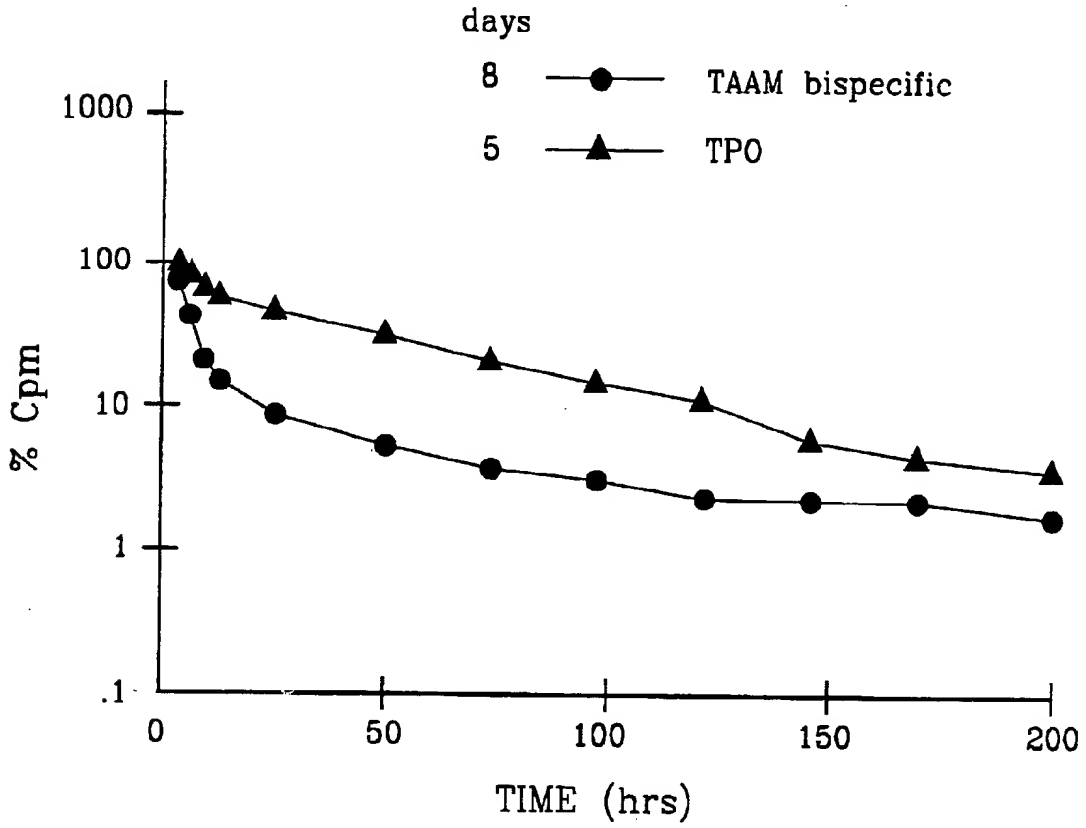
days
8
TAAM bispecific
5
TPO
1000
100
10
1
.1
% Cpm
0
50
100
150
200
TIME (hrs)

FIG. 4

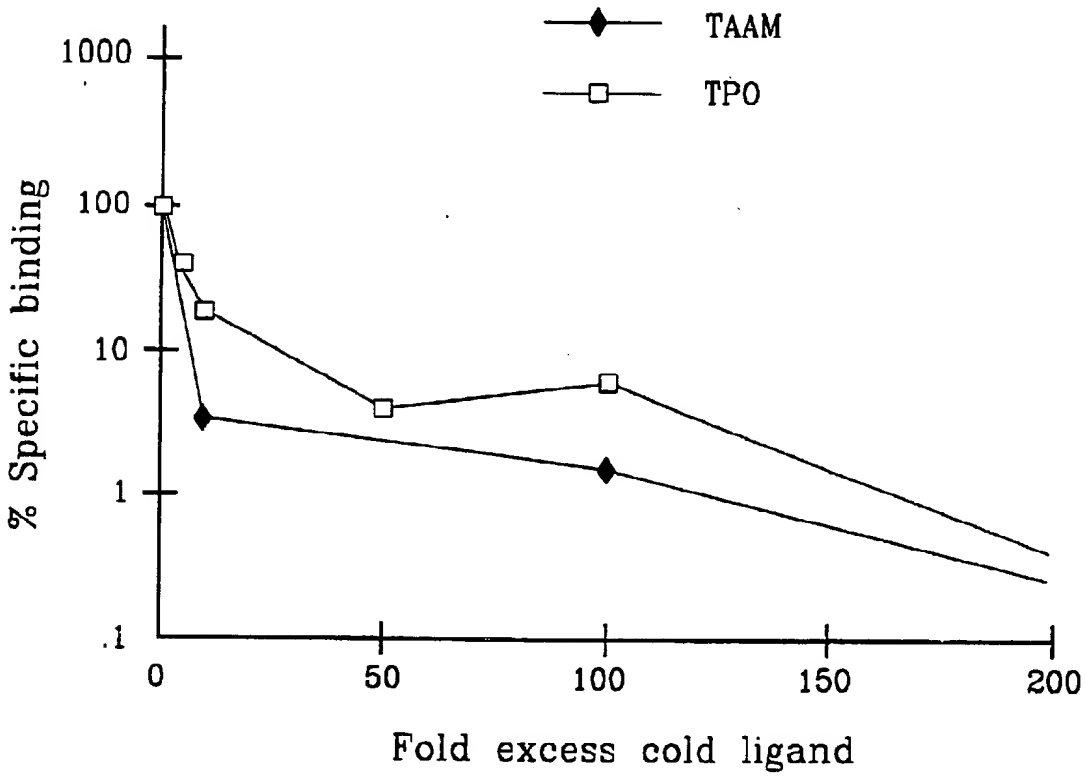
TAAM
TPO
1000
100
10
1
.1
% Specific binding
0
50
100
150
200
Fold excess cold ligand

FIG. 5

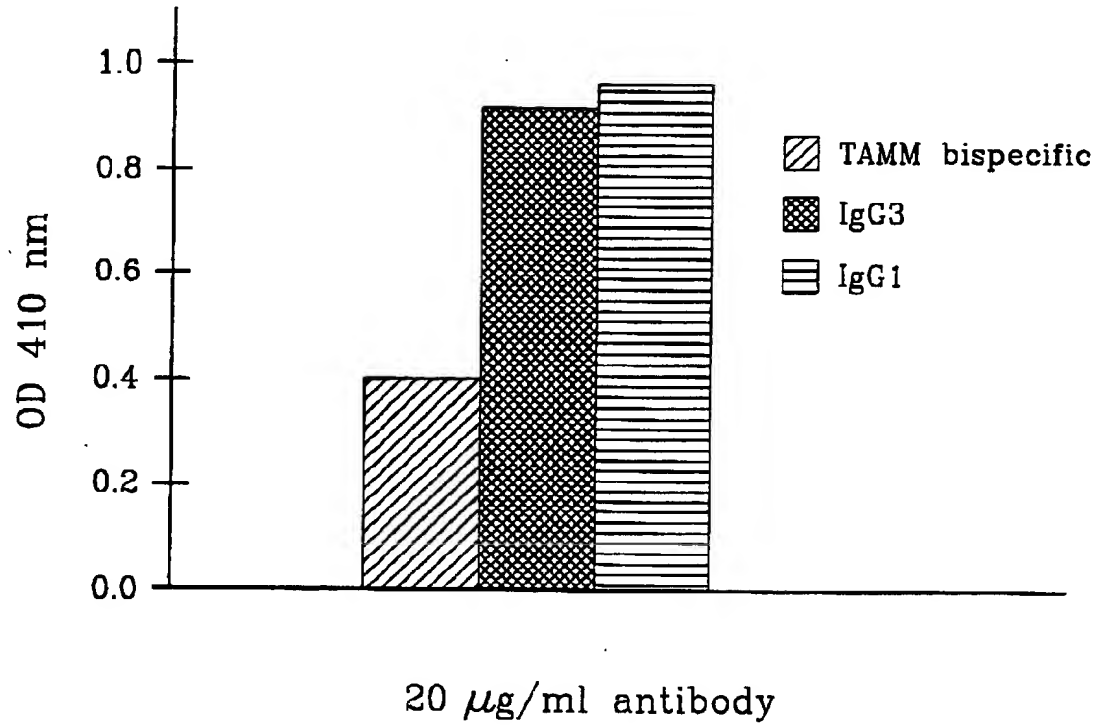
1.0
0.8
0.6
0.4
0.2
0.0
OD 410 nm
TAMM bispecific
IgG3
IgG1
20 μg/ml antibody

FIG. 6

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 94/11411

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/13 C07K16/46 C12N15/85 C12N5/10 G01N33/577
A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 517 024 (BEHRINGWERKE AG) 9 December 1992 see the whole document ---	1-31
A	EP,A,0 404 097 (BEHRINGWERKE AG) 27 December 1990 see the whole document ---	1-31
A	WO,A,93 11161 (ENZON INC.) 10 June 1993 see claims see figure 6B --- -/--	1-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 January 1995	20. 01. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	NOOIJ, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. ..onal Application No
PCT/US 94/11411

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	JOURNAL OF BIOTECHNOLOGY, vol.33, no.2, 1994, AMSTERDAM, THE NETHERLANDS pages 157 - 174 H. TADA ET AL. 'Expression and characterization of a chimeric bispecific antibody against fibrin and against urokinase-type plasminogen activator.' see the whole document ---	1-31
P,A	THERAPEUTIC IMMUNOLOGY, vol.1, no.1, January 1994, OXFORD, GB pages 3 - 15 M. HAYDEN ET AL. 'Single-chain mono- and bispecific antibody derivatives with novel biological properties and antitumour activity from a COS cell transient expression system.' see the whole document -----	1-31

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter..onal Application No
PCT/US 94/11411

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0517024	09-12-92	DE-A-	4118120	10-12-92
		AU-A-	1735692	10-12-92
		JP-A-	6128297	10-05-94
EP-A-0404097	27-12-90	DE-A-	3920358	17-01-91
		AU-B-	639241	22-07-93
		AU-A-	5762190	03-01-91
		CA-A-	2019559	22-12-90
		JP-A-	3048699	01-03-91
WO-A-9311161	10-06-93	AU-A-	3178993	28-06-93
		CA-A-	2122732	10-06-93
		EP-A-	0617706	05-10-94